J. Microbiol. Biotechnol. (2014), 24(4), 568–576
http://dx.doi.org/10.4014/jmb.1311.11077

jmb

Construction and Characterization of an Enhanced GFP-Tagged TIM-1 Fusion Protein

Jilin Qing[1], Haibing Xiao[2], Lin Zhao[3], Guifang Qin[3], Lihua Hu[4*], and Zhizhong Chen[3*]

[1]*Department of Gynecology, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning 530021, P.R. China*
[2]*Division of Neurology, Internal Medicine, The University of Hong Kong-Shenzhen Hospital, Shenzhen 518053, P.R. China*
[3]*Department of Laboratory, The People's Hospital of Guangxi Zhuang Autonomous Region, Nanning 530021, P.R. China*
[4]*Department of Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, P.R. China*

Received: November 25, 2013
Revised: January 11, 2014
Accepted: January 14, 2014

First published online
January 20, 2014

*Corresponding authors
Z.C.
Phone: +86-7712186127;
Fax: +86-7712186125;
E-mail: tjchenzz@163.com
L.H.
Phone: +86-2785726311;
Fax: +86-2785726311;
E-mail: tjxhhlh@163.com

pISSN 1017-7825, eISSN 1738-8872

TIM-1 (also known as KIM-1 and HAVcr-1) is a type I transmembrane glycoprotein member of the TIM family that may play important roles in innate and adaptive immune responses. The overexpression of proteins associated with membrane proteins is a major obstacle to overcome in studies of membrane protein structures and functions. In this study, we successfully coupled the overexpression of the TIM-1 protein with a C-terminal enhanced green fluorescent protein (GFP) tag in *Escherichia coli*. To the best of our knowledge, this report is the first to describe the overexpression of human TIM-1 in *E. coli*. The purified TIM-1-EGFP fusion protein recognized and bound directly to apoptotic cells and did not to bind to viable cells. Furthermore, we confirmed that the interactions of TIM-1-EGFP with apoptotic cells were blocked by TIM-1-Fc fusion proteins. This fusion protein represents a readily obtainable source of biologically active TIM-1 that may prove useful in future studies of human TIM-1.

Keywords: HAVcr-1, fusion protein, apoptosis, *E. coli*, membrane protein, EGFP

Introduction

Proteins of the T-cell immunoglobulin mucin (TIM) family are expressed by multiple cell types within the immune systems of rodents and humans [16]. The TIM family is composed of eight genes in mice, three of which are conserved in humans (TIM-1, TIM-3, and TIM-4) [24]. TIM proteins are type 1 membrane proteins that have a structurally conserved immunoglobulin variable (IgV) domain and a mucin stalk that connects to an intracellular tail [24]. TIM-1, also known as kidney injury molecule 1 (KIM-1) and human HAV cellular receptor 1 (HAVcr-1), is preferentially expressed on T-helper 2 (Th2) cells and functions as a potent costimulatory molecule for T-cell activation [3, 4]. In humans, TIM-1 also serves as a susceptibility gene for allergies and asthma [27]. TIM-3, which is expressed on T cells and dendritic cells, regulates T-cell apoptosis and immune tolerance [27, 32]. By contrast, TIM-4, which is expressed primarily on antigen-presenting cells and is a receptor for phosphatidylserine, regulates T-cell activation and tolerance [9, 27]. Recent studies have shown that a reduction in TIM-1 expression eliminates airway hyperreactivity that is normally triggered by the recognition of airway cell death [19]. Together, these studies suggest that the TIM family plays a critical role in immune responses and immune-mediated diseases.

Twenty to thirty percent of most proteomes are composed of membrane proteins [18]. These proteins play key roles in most biochemical pathways and disease, and they constitute more than half of all known drug targets [28]. However, membrane proteins, owing to their low abundance, are generally difficult to isolate in significant quantities from natural sources [25]. Therefore, the overexpression of membrane proteins is often essential for structural and

functional studies. Recent studies support the notion that green fluorescent protein (GFP) fusion proteins provide a potentially facile tool for the identification of well-expressed, properly behaved, membrane proteins for their biochemical and structural study [11]. Enhanced GFP (EGFP) is a mutant of GFP with a 35-fold increase in fluorescence [31]. The human TIM-1 gene is predicted to encode a type I membrane protein and is an important regulator of immune responses [3]. To better understand the role of TIM-1 in apoptosis and immune regulation, in the present study, we describe the construction of the vector pET28a-TIM-1-EGFP, which was derived from the vector pET28a and was fused at the C-terminus with EGFP, for the overexpression of the TIM-1 eukaryotic membrane protein in *Escherichia coli*.

Materials and Methods

Construction of the TIM-1-EGFP Fusion Protein Expression Vector

Total RNA was extracted from human peripheral blood mononuclear cells (PMBC) according to the Trizol reagent's instructions (Invitrogen, USA). The cDNA library of the human TIM-1 gene was constructed by reverse transcription with the manufacturer's protocol (Takara Biotechnology (Dalian Co., Ltd.), China). To construct the expression plasmids of human TIM-1, the cDNA fragment that encodes human TIM-1 (excluding the stop codon) was prepared by RT-PCR. The PCR primers were 5-CATGCCATGGATATGCATCCTCAAGTGGTCATCT-3 (the *Nco*I cloning site is underlined) and 5-GCGAGCTCGTCCGTGGCA TAAAGACTATTCTC-3 (the *Sac*I cloning site is underlined). The cDNA was subcloned into the *Nco*I and *Sac*I sites of pET28-a (Novagen, Germany). The constructed plasmid was designated as pET28a-TIM-1 and confirmed by conventional PCR and sequence analysis. The EGFP sequence was amplified from the vector pEGFP-C2 by using the sense primer 5-GCGAGCTCATGGTGA GCAAGGGCG-3 (the *Sac*I cloning site is underlined) and the antisense primer 5-CCGCTCGAGCTTGTACAGCTCGTCCATGC-3 (the *Xho*I cloning site is underlined). The amplified EGFP fragment was ligated into the pMD18-T simple vector and, following sequencing, the fragment was subcloned into the *Sac*I and *Xho*I sites of pET28a-TIM-1, which resulted in the addition of a C-terminal EGFP-His6 tag. In addition, the same fragment was subcloned into the *Sac*I and *Xho*I sites of pET28a to create a control plasmid. The constructed plasmids were designated pET28a-TIM-1-EGFP (Fig. 1) and pET28a-EGFP, respectively. The plasmids were then confirmed by PCR and an endonuclease restriction digestion assay.

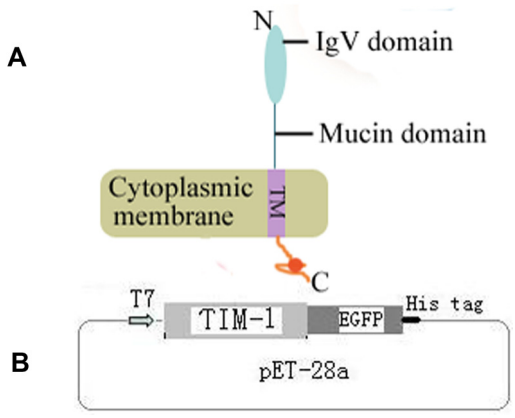

Fig. 1. Structure of the human TIM-1 gene and the expression vector of the TIM-1-EGFP fusion protein.
(**A**) Structure of the human TIM-1 gene. (**B**) The pET28a-TIM-1-EGFP expression vector.

Expression and Purification of Fusion Protein

To express and purify the TIM-1-EGFP fusion protein, the pET28a-TIM-1-EGFP recombinant plasmid was transformed into a competent *E. coli* BL21(DE3) strain. A single colony was picked from a selective plate, inoculated into LB medium containing 50 μg/ml kanamycin, and was then incubated overnight at 37°C with shaking (190 rpm). The cultures were diluted 1:50 into a selective LB medium and were then allowed to grow at 37°C with vigorous shaking (220 rpm) until the optical density (OD_{600}) reached 0.6. Next, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM for 18 h at 30°C, and the bacterial cells were harvested by centrifugation at 6,000 ×*g* for 15 min at 4°C. Cell pellets were washed with cold PBS, centrifuged, and resuspended in 50 ml of lysis buffer (20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 10% glycerol), and 1 mg/ml lysozyme (final concentration) was added to the lysis buffer and incubated for 30 min on ice. Cells were then lysed on ice by sonication. Finally, 10% Triton X-100 was added for a final concentration of 0.05%, and the solution was mixed and incubated on ice for 15 min. During cell lysis, protease inhibitor PMSF was added to a final concentration of 1 mM. The supernatant was collected by centrifugation at 10,000 ×*g* for 30 min at 4°C. The resulting supernatant was passed through a 0.45 μm syringe-end filter, and purification was carried out under native conditions using Ni-NTA resin (BBI Biotech, UK) according to the manufacturer's instructions. In brief, we equilibrated the column with 10 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0), and added the sample. Next, we washed the column with 10 ml of binding buffer. Lastly, we eluted the column with 4 ml of elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole (pH 8.0)) and collected the eluates into four separate 1.5 ml Eppendorf tubes in chronological order (1.0 ml per tube). The purity of the recombinant fusion proteins was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed according to the instructions of the SDS-PAGE Gel Preparation Kit (Beyotime,

China), and the proteins were detected by Coomassie Brilliant Blue R-250 staining and western blotting analysis. Quantification of the purified recombinant TIM-1-EGFP fusion protein was performed by BCA kit (Beyotime, China).

Microscopic Observation

To evaluate the expression of TIM-1-EGFP fusion protein, bacterial cultures were spotted on a microscope slide and mounted with glycerol. Subsequently, samples were observed under a fluorescence microscope.

Cell Culture

K-562, PANC-1, HepG2, and CHO cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Pervanadate Preparation

Sodium orthovanadate (activated) stock solution was prepared by adding sodium orthovanadate to purified water for a concentration of 200 mM, and the activation of sodium orthovanadate was performed as has been previously described [10]. Pervanadate (PV) was freshly prepared in each experiment as follows: 150 µl of 200 mM sodium orthovanadate was placed in an Eppendorf tube containing 843 µl of phosphate-buffered saline and was treated with 7 µl of 30% H_2O_2 followed by incubation in the dark for 15 min at room temperature. Excess H_2O_2 was deactivated with the addition of 5 µl of catalase (200 mg/ml), which was allowed to incubate in the dark for 5 min at room temperature. PV stock (30 mM) was diluted with the existing medium on the cells for a resulting final concentration of 100 µM PV.

Analysis of Apoptosis by Fluorescence Microscopy

K-562, PANC-1, HepG2, and CHO cell lines were maintained in RPMI 1640 medium containing 10% fetal bovine serum. To induce apoptosis in the cultured cells, the cells were grown in a cell culture bottle with or without 100 µM pervanadate for 12 h (K562 cells for 24 h) or with 200 µM H_2O_2 for 12 h (K562 cells for 24 h). After pervanadate or H_2O_2 treatment, human K562 cells (2×10^5 cells) were collected by centrifugation at 2,000 rpm for 5 min. PANC-1, HepG2, and CHO cells (2×10^5 cells) were harvested using trypsin without EDTA. After the cells were digested, they were kept in culture medium with serum to prevent overdigestion. The PANC-1, HepG2, and CHO cells were collected by centrifugation at 2,000 rpm for 5 min. The K-562, PANC-1, and HepG2 cells were rinsed twice with PBS and harvested by centrifugation at 2,000 rpm for 5 min and the cells were resuspended in 500 µl of culture medium containing 2% serum. The cells were combined with 6 µl of the eluate (TIM-1-EGFP) and mixed in the dark for 15 min. After incubation with TIM-1-EGFP, the cells were centrifuged at 1,500 rpm for 5 min and the supernatant was discarded. Next, the cells were gently resuspended in 500 µl of culture medium containing 2% fetal bovine serum and were then observed by fluorescence microscopy. Apoptosis (and for the control) was detected using a commercially available annexin V kit (KeyGen, China) according to the manufacturer's instructions.

TIM-1-Fc Fusion Protein

The TIM-1-Fc fusion protein consists of the complete TIM-1 extracellular domain linked to the hinge-CH_2-CH_3 domains of human IgG1 in the pcDNA3.1(+) vector. In brief, the complete TIM-1 extracellular domain was amplified with a 5′ primer (5-CTAGCTAGCGCCACCATGCATCCTCAAGTGGTCATCT-3) and a 3′ primer (5-GGGGTACCTCCTTTAGTGGTATTGGCCGT-3), which contained unique *Nhe*I and *Kpn*I restriction sites at the 5′ and 3′ ends, respectively. The Fc portion of human IgG1 was amplified with a 5′ primer (5-GGGGTACCGAGCCCAAATCTT GTGACAAAACT-3) and a 3′ primer (5-CCGCTCGAGTCATTT ACCCGGAGACAGGGA-3), which contained unique *Kpn*I and *Xho*I restriction sites at the 5′ and 3′ ends, respectively. The amplification product of the TIM-1 ectodomain and the IgG1 Fc fragment were each cloned into the eukaryote expression vector pcDNA3.1(+). All DNA constructs were confirmed by automated DNA sequencing. The pcDNA3.1(+)TIM-1-Fc plasmid was transiently transfected into CHO cells according to the instructions of the Lipofectamine 2000 kit (Invitrogen, USA). The expression of TIM-1-Fc was confirmed by RT-PCR and western blot analysis. The supernatants were harvested at 48 and 72 h after transfection and were stored at −20°C or −70°C for additional experimentation.

***In Situ* Detection of Apoptosis**

CHO and HepG2 cells, which were cultured in a 24-well plate, were maintained in RPMI 1640 medium containing 10% fetal bovine serum. To induce apoptosis of the cultured cells, CHO and HepG2 cells were incubated for 12 h at 37°C and 5% CO_2 in complete medium alone or that containing 100 µM PV. After PV treatment, 5.5 µl of the purified eluate (TIM-1-EGFP) was added to cells in the existing medium and incubated in the incubator for 1 h in the dark. However, the HepG2 cells were pretreated for 1 h with 200 µl supernatants of the 48 h harvest that contained TIM-1-Fc protein. After 1 h of incubation, cells were washed three times with PBS buffer and were then observed by fluorescence microscopy.

Results

Construction of Expression Vector pET28a-TIM-1-EGFP

The pET28a vector, which contains a C-terminal 6×His tag, was used for the subcloning of the TIM-1 and EGFP genes. The C-terminal EGFP tag, following the His6 sequence downstream of EGFP for Ni-NTA purification, was used to facilitate the monitoring of membrane protein overexpression. The resulting vector, designated pET28a-TIM-1-EGFP, was used to achieve the overproduction of the eukaryotic membrane protein TIM-1 (Fig. 1).

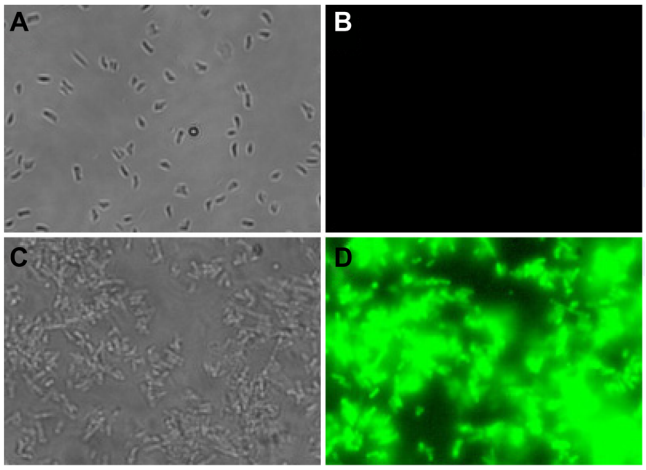

Fig. 2. Microscopic evaluation of protein expression.
Mounted cells were observed with a fluorescence microscope. (**A**) Bacterial clone uninduced (phase contrast). (**B**) The same clone as in (**A**) (fluorescence). (**C**) Bacteria expressing a TIM-1-EGFP fusion protein (induced by 1.0 mM IPTG, 30°C, 18 h) (phase contrast). (**D**) The same image as in (**C**) (fluorescence).

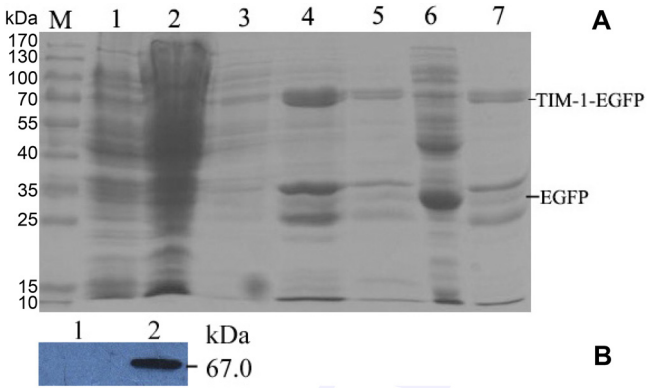

Fig. 3. Expression of TIM-1-EGFP fusion protein upon induction.
(**A**) SDS-PAGE analysis of TIM-1-EGFP by Coomassie Blue R-250 staining. Lane **M**, PageRuler Prestained Protein Ladder (SM0671, Fermentas); lane **1**, whole-cell lysates of uninduced bacteria (BL21-pET28a-TIM-1-EGFP cells); lane **2**, whole-cell lysates of uninduced bacteria (BL21-pET28a-EGFP cells); lane **3**, purified TIM-1-EGFP in the fourth 1 ml elution; lane **4**, purified TIM-1-EGFP in the second 1 ml elution; lane **5**, purified TIM-1-EGFP in the first 1 ml elution; lane **6**, whole-cell lysates of IPTG-induced bacteria (BL21-pET28a-EGFP cells); and lane **7**, purified TIM-1-EGFP in the third 1 ml elution. (**B**) Western blot analysis of TIM-1-EGFP. Lanes **1** and **2** represent the soluble lysate of BL21-pET28a-EGFP and the purified eluate of BL21-pET28a-TIM-1-EGFP cells, respectively, followed by western blot analysis (detailed in Fig. 3B) using a MAb against the His6-tag (Tiangen, China).

Expression and Purification of Fusion Protein

E. coli is the most widely used vehicle for the overexpression of eukaryotic membrane proteins. The pET28a-TIM-1-EGFP plasmid was subsequently transformed to *E. coli* BL21(DE3). To confirm the expression of TIM-1-EGFP after induction, transformed TIM-1-EGFP cells were examined by fluorescence microscopy. Fluorescent signals in induced BL21(DE3) cells expressing TIM-1-EGFP are shown in Fig. 2. Compared with the uninduced control, the induced bacteria displayed a significant GFP signal. The TIM-1-EGFP fusion protein was purified using the Ni-NTA resin application, and fusion protein purity was confirmed by SDS-PAGE and Coomassie Blue staining. A predominant protein with a mass of 67.0 kDa was observed (TIM-1 protein, 39.25 kDa; EGFP protein, 27 kDa; TIM-1-EGFP fusion protein containing the His tag) (Fig. 3A), which is consistent with the calculated molecular mass of the fusion protein, and almost no basal expression was observed in the uninduced cultures. The TIM-1-EGFP fusion protein was generally collected in the second 1 ml eluate. Furthermore, the identity of this band as the TIM-1-EGFP fusion protein was confirmed by western blot analysis using a mouse anti-His6-tag (Fig. 3B) IgG Ab and an HRP-conjugated goat anti-mouse IgG Ab. The yield of the purified protein was 1.20 mg/l as measured by BCA assay.

Expression of TIM-1-Fc Fusion Protein in CHO Cells

A 1,616 bp fragment was amplified from the total RNA of TIM-1-Fc/CHO cells by RT-PCR, corresponding to the anticipated size. However, RT-PCR failed to amplify the target sequence using cDNAs derived from transfected empty pcDNA3.1(+) vector CHO cells (Fig. 4A). Western blot analysis of the culture medium using an anti-human IgG1Fc antibody detected a 57.6 kD protein, which is in agreement with the molecular mass of TIM-1-Fc fusion protein (Fig. 4B).

Biological Activity of TIM-1-EGFP

Kobayashi *et al.* [20] showed that TIM-1 and TIM-4 glycoproteins bound phosphatidylserine and mediated the uptake of apoptotic cells. Following the purification of fusion protein TIM-1-EGFP, we next verified the binding activity of TIM-1-EGFP to apoptotic cells. Pervanadate, a potent inhibitor of tyrosine phosphatases, was used to induce apoptosis of human K562, HepG2, and PANC-1 cells. PV-induced apoptotic cell shrinkage and pyknosis were visible by light microscopy, and these cells were smaller in size (data not shown).

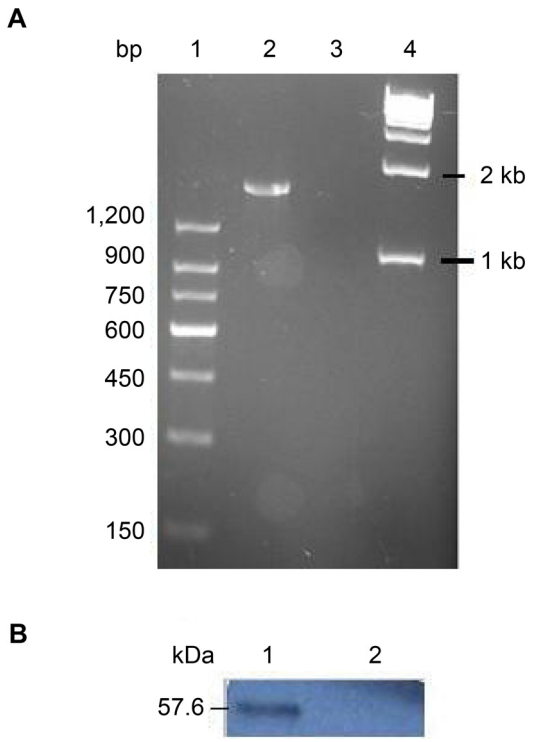

Fig. 4. Identification of TIM-1-Fc mRNA and fusion protein expression.

(**A**) Analysis of the expression of TIM-1-Fc mRNA in CHO cells by RT-PCR. Lane **1**: 150 bp DNA Ladder Marker; lane **2**: the RT-PCR result of the CHO cells transfected with the TIM-1-Fc fusion plasmids (1,616 bp); lane **3**: the RT-PCR result of the CHO cells transfected with the pcDNA3.1(+) plasmids; lane **4**: 1 kb DNA Ladder Marker. (**B**) Detection of TIM-1-Fc fusion protein in cell culture supernatant by western blot. Lane **1**: culture supernatant of CHO cells with pcDNA3.1(+)TIM-1-Fc plasmid; lane **2**: culture supernatant of CHO cells with the pcDNA3.1(+) plasmid.

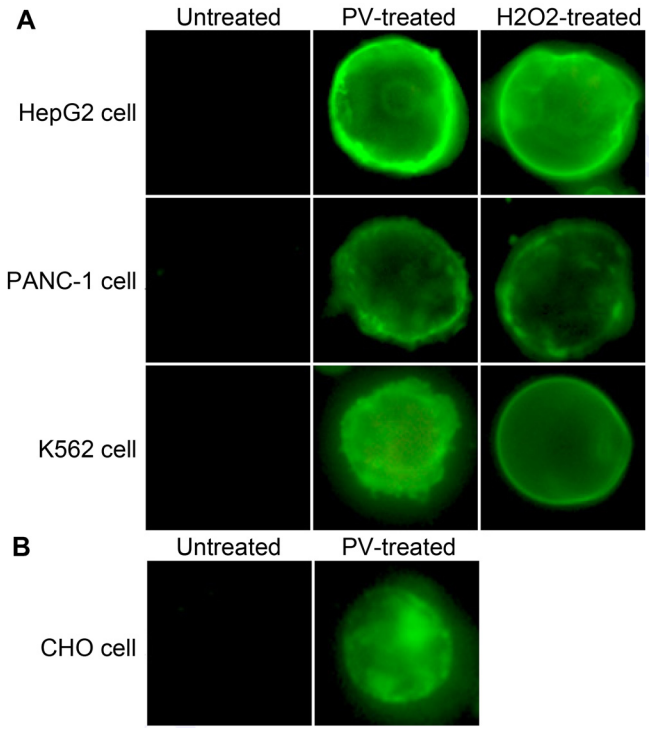

Fig. 5. Early-phase apoptosis detection through annexin V staining.

(**A**) The 100 μM PV and 200 μM H_2O_2 induced the apoptosis of K-562 and PANC-1 as well as HepG 2 cells, respectively. Untreated cells are not labeled, and the apoptotic cells labeled with annexin V-FITC are visualized in green. (**B**) Apoptosis of CHO cells induced by 100 μM of PV. Untreated CHO cells are not labeled, and treated apoptotic CHO cells are visualized in green with annexin V-FITC.

One of the earliest apoptotic features in cells is phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane. Following the described treatment, annexin V in parallel with TIM-1-EGFP staining, the results of the binding of annexin V-FITC to cells is visualized in Fig. 5. Once the apoptotic cells are bound with TIM-1-EGFP fusion proteins, they can be visualized by fluorescence microscopy. As shown in Fig. 6, the untreated K562 cells had no fluorescence; the untreated HepG2 and PANC-1 cells had very little nonspecific fluorescence, which may have been caused by poor trypsin digestion. In contrast to the untreated cells, after the treatment of pervanadate or H_2O_2, the K562, HepG2, and PANC-1 cells exhibited bright green fluorescence. These results show that TIM-1-EGFP fusion proteins recognize and bind directly to apoptotic cells and not to viable cells. Moreover, we combined TIM-1-EGFP with PI staining for the analysis of cells undergoing apoptosis. As shown in Fig. 7, the early-staged apoptotic cells stained green with TIM-1-EGFP and negatively with PI, and the late-stage apoptotic or necrotic cells stained green with TIM-1-EGFP and red with PI.

To further characterize the binding of TIM-1-EGFP to apoptotic cells, we prepared Ig fusion proteins with the extracellular domains of TIM-1. As shown in Fig. 8, the untreated CHO and HepG2 cells had very little nonspecific fluorescence, which may have been caused by the long staining period or by inadequate washing. Without treatment of TIM-1-Fc fusion proteins, the PV-treated CHO and HepG2 cells exhibited bright green fluorescence. By contrast, with a pretreatment of TIM-1-Fc fusion protein, the PV-treated CHO and HepG2 cells exhibited very little fluorescence. These findings suggest that the interactions of

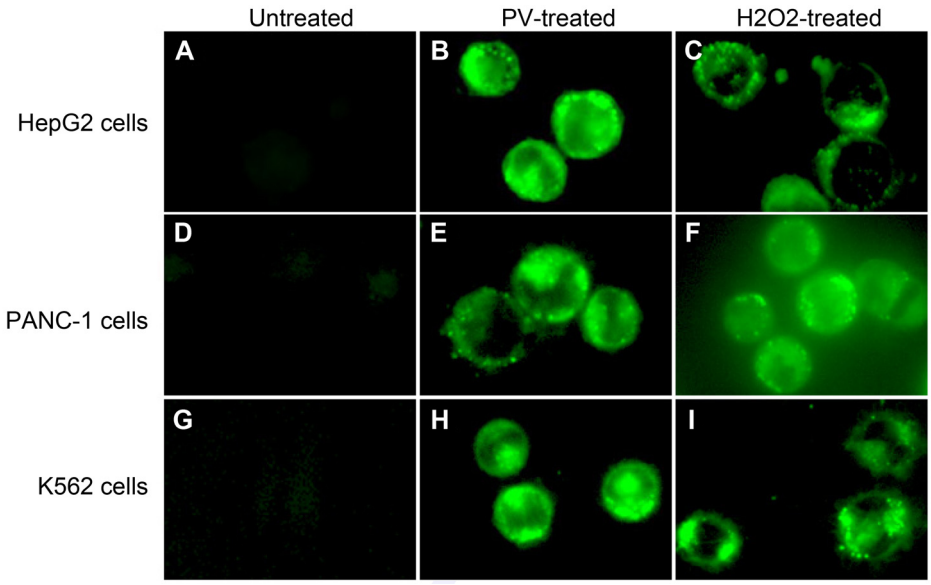

Fig. 6. Effects of purified TIM-1-EGFP fusion protein on apoptotic cells *in vitro*.
The untreated K562 cells (**G**) had no fluorescence; the untreated HepG2 (**A**) and PANC-1 cells (**D**) had very little nonspecific fluorescence. In contrast to the untreated cells, following a treatment of pervanadate or H_2O_2, K562 (**H**, **I**), HepG2 (**B**, **C**), and PANC-1 cells (**E**, **F**) exhibited bright green fluorescence.

TIM-1-EGFP with apoptotic cells were blocked by TIM-1-Fc fusion proteins. Therefore, human TIM-1 may mediate the recognition and binding of apoptotic cells and play a role in the regulation of apoptosis.

Discussion

TIM-1, an important susceptibility gene for asthma and allergies, is preferentially expressed on T-helper 2 cells and functions as a potent costimulatory molecule for T-cell activation [3]. TIM-1 was originally discovered in African green monkey kidney cells, and later in humans as a hepatitis A virus cellular receptor (HAVcr), which is the receptor exploited by hepatitis A for viral entry [8, 17]. Moreover, binding of the hepatitis A virus to cellular receptor 1 inhibits T-regulatory cell functions in humans [23]. A recent study also suggested that TIM-1 mediates virus entry and virulence [21]. TIM-1 was also previously identified as a marker that is upregulated on the surfaces of injured kidney cell epithelia in acute kidney injury models of rats and humans [12, 15, 26]. Recent research has revealed that sustained KIM-1 expression promotes kidney fibrosis and provides a link between acute and recurrent injuries with progressive chronic kidney disease [13].

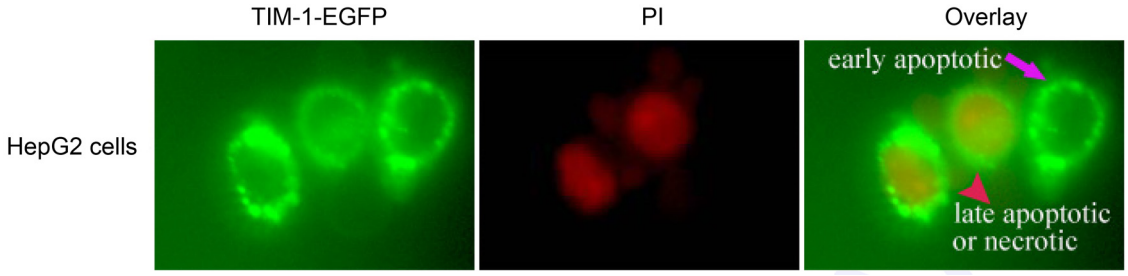

Fig. 7. PV-induced binding of TIM-1-EGFP/PI to HepG2 cells *in situ*.
The early-staged apoptotic HepG2 cells were stained green with TIM-1-EGFP and negatively with PI; the late-staged apoptotic or necrotic HepG2 cells were stained green with TIM-1-EGFP, and red with PI.

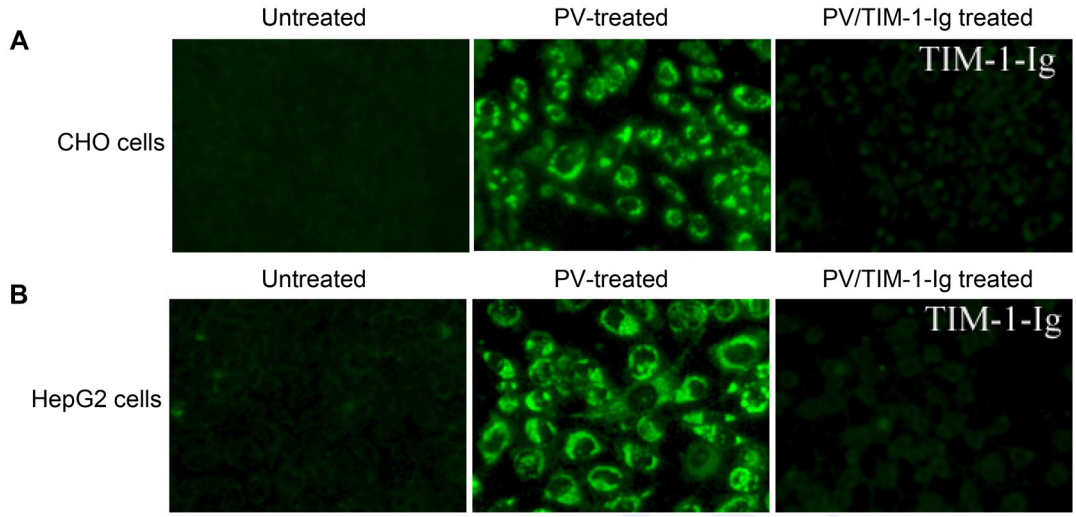

Fig. 8. TIM-1-Fc fusion protein blockade and TIM-1-EGFP binding to apoptotic cells.
(**A**) CHO cells that were untreated (left panel), PV-treated (middle panel), or preincubated with TIM-1-Fc of PV-treated CHO cells (right panel) were stained *in situ* with TIM-1-EGFP. (**B**) HepG2 cells untreated (left panel), PV-treated (middle panel), or preincubated with TIM-1-Fc of PV-treated HepG2 cells (right panel) were stained *in situ* with TIM-1-EGFP.

Furthermore, TIM-1 may also have a utility as a cancer marker because it is upregulated in tissue samples of patients with renal cell carcinoma or colorectal cancer [2, 22, 29, 30]. Several recent studies have shown that TIM-1 specifically bound phosphatidylserine on the surface of apoptotic cells and may mediate the uptake of apoptotic cells [14, 20]. Taken together, TIM-1 may play a key role in immune tolerance and regulation.

Membrane proteins play important roles in many diseases, and more than half of all drug targets are membrane proteins [28]. The natural abundance of most membrane proteins is usually too low to isolate sufficient material for functional and structural studies [1]. Therefore, membrane proteins must be obtained by overexpression, which can be an obstacle in studies of membrane protein function and structure [1]. Recently, advances have been made in developing methods that can assess the expression and purification of membrane proteins in a high-throughput manner. To date, the most widely used method to monitor such expression levels is the fusion of green fluorescent protein to the C-terminus of the target membrane protein [6, 7]. Human TIM-1 is a 364 amino acid protein containing a putative signal sequence and a transmembrane domain [3]. To better explore the role of TIM-1 in the context of immune regulation and immune-mediated diseases, we constructed a pET28a-TIM-1-EGFP vector with a C-terminal EGFP-His6 tag. The EGFP-moiety allowed direct monitoring of the protein during all steps from expression to purification, while the histidine tag expressed on the protein facilitated capture of the protein of interest by a Ni-NTA column. *E. coli* is one of the most widely used vehicles to overexpress both prokaryotic and eukaryotic (membrane) proteins because *E. coli* is very well accessible and easy to handle [5]. Our results show that the TIM-1 membrane protein fused to EGFP is successfully overexpressed from a pET28a-derived histidine tag vector in *E. coli* BL21(DE3).

After the purification of fusion protein TIM-1-EGFP, we next explored whether the TIM-1 membrane protein was functional or not. Kobayashi *et al.* [20] showed that TIM-1 and TIM-4 glycoproteins bound phosphatidylserine and mediated the uptake of apoptotic cells. To evaluate the binding activity of TIM-1-EGFP to apoptotic cells, we used TIM-1-EGFP as a probe to stain the apoptotic cells. Once the apoptotic cells are bound with TIM-1-EGFP fusion proteins, they can be visualized by fluorescence microscopy. Our results demonstrated that TIM-1-EGFP fusion proteins recognize and bind directly to apoptotic cells and do not bind to viable cells. Moreover, we confirmed that the interactions of TIM-1-EGFP with apoptotic cells are blocked by TIM-1-Fc fusion proteins. These findings suggest that human TIM-1 may mediate the regulation of apoptosis. Interestingly, in the present study, we used three tumor cell lines as apoptotic cells models and verified that the human TIM-1 protein can interact with the PV-induced

apoptotic cells. This result suggests that human TIM-1 may be related to the clearance of the apoptotic tumor cells.

In conclusion, we successfully constructed a fusion protein consisting of fluorescence-enhanced green fluorescent protein (EGFP) and human TIM-1 and expressed this protein in *E. coli* BL21(DE3). Our results also demonstrate that the TIM-1-EGFP fusion protein mediates the recognition and binding to apoptotic cells. Thus, the fusion protein represents a readily obtainable source of biologically active TIM-1 that may prove useful in future studies of human TIM-1.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81260007 and 30672008).

References

1. Carpenter EP, Beis K, Cameron AD, Iwata S. 2008. Overcoming the challenges of membrane protein crystallography. *Curr. Opin. Struct. Biol*. **18:** 581-586.
2. Cuadros T, Trilla E, Vila MR, de Torres I, Vilardell J, Messaoud NB, *et al*. 2013. Hepatitis A virus cellular receptor 1/kidney injury molecule-1 is a susceptibility gene for clear cell renal cell carcinoma and hepatitis A virus cellular receptor/kidney injury molecule-1 ectodomain shedding a predictive biomarker of tumour progression. *Eur. J. Cancer* **49:** 2034-2047.
3. Curtiss M, Colgan J. 2007. The role of the T-cell costimulatory molecule Tim-1 in the immune response. *Immunol. Res*. **39:** 52-61.
4. Curtiss ML, Gorman JV, Businga TR, Traver G, Singh M, Meyerholz DK, *et al*. 2012. Tim-1 regulates Th2 responses in an airway hypersensitivity model. *Eur. J. Immunol*. **42:** 651-661.
5. Drew D, Froderberg L, Baars L, de Gier JW. 2003. Assembly and overexpression of membrane proteins in *Escherichia coli*. *Biochim. Biophys. Acta* **1610:** 3-10.
6. Drew D, Lerch M, Kunji E, Slotboom DJ, de Gier JW. 2006. Optimization of membrane protein overexpression and purification using GFP fusions. *Nat. Methods* **3:** 303-313.
7. Drew DE, von Heijne G, Nordlund P, de Gier JW. 2001. Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli*. *FEBS Lett*. **507:** 220-224.
8. Feigelstock D, Thompson P, Mattoo P, Zhang Y, Kaplan GG. 1998. The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor. *J. Virol*. **72:** 6621-6628.
9. Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. 2010. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. *Immunol. Rev*. **235:** 172-189.
10. Gordon JA. 1991. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol*. **201:** 477-482.
11. Hammon J, Palanivelu DV, Chen J, Patel C, Minor Jr DL. 2009. A green fluorescent protein screen for identification of well-expressed membrane proteins from a cohort of extremophilic organisms. *Protein Sci*. **18:** 121-133.
12. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. 2002. Kidney injury molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int*. **62:** 237-244.
13. Humphreys BD, Xu F, Sabbisetti V, Grgic I, Naini SM, Wang N, *et al*. 2013. Chronic epithelial kidney injury molecule-1 expression causes murine kidney fibrosis. *J. Clin. Invest*. **123:** 4023-4035.
14. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. 2008. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J. Clin. Invest*. **118:** 1657-1668.
15. Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M. 1998. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *J. Biol. Chem*. **273:** 4135-4142.
16. Kane LP. 2010. T cell Ig and mucin domain proteins and immunity. *J. Immunol*. **184:** 2743-2749.
17. Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone SM. 1996. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. *EMBO J*. **15:** 4282-4296.
18. Keating AE. 2007. A rational route to probing membrane proteins. *Genome Biol*. **8:** 214.
19. Kim HY, Chang YJ, Chuang YT, Lee HH, Kasahara DI, Martin T, *et al*. 2013. T-cell immunoglobulin and mucin domain 1 deficiency eliminates airway hyperreactivity triggered by the recognition of airway cell death. *J. Allergy Clin. Immunol*. **132:** 414-425.e6.
20. Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, *et al*. 2007. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity* **27:** 927-940.
21. Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL, Moller-Tank S, *et al*. 2011. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. *Proc. Natl. Acad. Sci. USA* **108:** 8426-8431.
22. Lin F, Zhang PL, Yang XJ, Shi J, Blasick T, Han WK, *et al*. 2007. Human kidney injury molecule-1 (hKIM-1): a useful immunohistochemical marker for diagnosing renal cell carcinoma and ovarian clear cell carcinoma. *Am. J. Surg. Pathol*. **31:** 371-381.

23. Manangeeswaran M, Jacques J, Tami C, Konduru K, Amharref N, Perrella O, *et al*. 2012. Binding of hepatitis A virus to its cellular receptor 1 inhibits T-regulatory cell functions in humans. *Gastroenterology* **142:** 1516-1525.e3.
24. Meyers JH, Sabatos CA, Chakravarti S, Kuchroo VK. 2005. The TIM gene family regulates autoimmune and allergic diseases. *Trends Mol. Med*. **11:** 362-369.
25. Neophytou I, Harvey R, Lawrence J, Marsh P, Panaretou B, Barlow D. 2007. Eukaryotic integral membrane protein expression utilizing the *Escherichia coli* glycerol-conducting channel protein (GlpF). *Appl. Microbiol. Biotechnol*. **77:** 375-381.
26. Nozaki Y, Nikolic-Paterson DJ, Yagita H, Akiba H, Holdsworth SR, Kitching AR. 2011. Tim-1 promotes cisplatin nephrotoxicity. *Am. J. Physiol. Renal Physiol*. **301:** F1098-F1104.
27. Rodriguez-Manzanet R, DeKruyff R, Kuchroo VK, Umetsu DT. 2009. The costimulatory role of TIM molecules. *Immunol. Rev*. **229:** 259-270.
28. Tan S, Tan HT, Chung MC. 2008. Membrane proteins and membrane proteomics. *Proteomics* **8:** 3924-3932.
29. Vila MR, Kaplan GG, Feigelstock D, Nadal M, Morote J, Porta R, *et al*. 2004. Hepatitis A virus receptor blocks cell differentiation and is overexpressed in clear cell renal cell carcinoma. *Kidney Int*. **65:** 1761-1773.
30. Wang Y, Martin TA, Jiang WG. 2013. HAVcr-1 expression in human colorectal cancer and its effects on colorectal cancer cells *in vitro*. *Anticancer Res*. **33:** 207-214.
31. Yang TT, Cheng L, Kain SR. 1996. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. *Nucleic Acids Res*. **24:** 4592-4593.
32. Zhu C, Anderson AC, Kuchroo VK. 2011. TIM-3 and its regulatory role in immune responses. *Curr. Top. Microbiol. Immunol*. **350:** 1-15.